federal register

FRIDAY, MARCH 31, 1978
PART III

DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE

National Institutes of Health

U.S.—EMBO Workshop to Assess Risks for Recombinant DNA Experiments Involving the Genomes of Animal, Plant, and Insect Viruses

[4110-08]

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Report

On September 27, 1977, proposed revised Guidelines for Research Involving Recombinant DNA Molecules were published in the FEDERAL REGISTER (42 FR 49596 et seq.) for public comment and consideration. The proposed revised Guidelines and comments received were considered by the Advisory Committee to the Director, National Institutes of Health (NIH), at its meeting on December 15-16, 1977.

In response to discussion concerning viruses at the Director's Advisory Committee meeting, a joint U.S.—EMBO Workshop to Assess Risks for Recombinant DNA Experiments Involving the Genomes of Animal, Plant, and Insect Viruses was held in Ascot, England, on January 26-28, 1978. The workshop was attended by 27 scientists from the United States, the United Kingdom, West Germany, Finland, France, Sweden, and Switzerland. The participants were invited because of their scientific expertise and not as representatives of any government or of any policymaking group.

The primary purpose of the meeting was to conduct a scientific and technical analysis of possible risks associated with cloning eukaryotic viral DNA segments in *E. coli* K-12 host-vector systems and with the use of eukaryotic viruses as cloning vectors in animal, plant, and insect systems. In addition, there were general discussions of the possible importance of recombinant DNA technology for the solution of problems in basic and applied virology and of the classification of viruses with respect to the hazard that laboratory research with them might pose to the laboratory worker or to the community.

To provide further opportunity for public comment and consideration, the Workshop report is presented below. This report will be considered by the Recombinant DNA Molecule Program Advisory Committee at its meeting on April 27-28, 1978. On the basis of comments received and review by the Recombinant Advisory Committee, the Director, NIH, will subsequently issue revised Guidelines for Research Involving Recombinant DNA Molecules accompanied by a decision document explaining the modifications.

Please address any comments on this report to the Director, National Institutes of Health, 9000 Rockville Pike, Bethesda, Md. 20014. All comments should be received by April 15, 1978. Additional copies of this notice are available from:

Director, Office of Recombinant DNA Activities, Building 31, Room 4A52, National Institutes of Health, 9000 Rockville Pike, Bethesda, Md. 20014.

Dated: March 22, 1978.

DONALD S. FREDERICKSON,
Director,
National Institutes of Health.

REPORT OF U.S.-EMBO WORKSHOP TO ASSESS RISKS FOR RECOMBINANT DNA EXPERIMENTS INVOLVING THE GENOMES OF ANIMAL, PLANT, AND INSECT VIRUSES

This is the report of a joint U.S.-EMBO Workshop held in Ascot, England, January 27-29, 1978, which was convened to discuss the possible risks of recombinant DNA experiments involving the DNA's of animal, plant, and insect viruses. The 27 scientists in attendance (see attached roster) had expertise in clinical infectious disease; public health, medical and diagnostic virology; the biology of virus infection; biochemical virology; and plant, insect, and veterinary viruses. Five of the participants are actively engaged in recombinant DNA experimentation. A consensus statement of the discussions in the areas of pathogenesis and epidemiology of viral diseases, potential benefits of recombinant DNA experiments involving eukaryotic viral DNA, viral hazard classifications, and cloning in prokaryotic and eukaryotic systems is presented below. The group's conclusions, with respect to possible risks of recombinant DNA experiments involving viruses are based on the best available scientific data derived from publications, knowledge of current activities in the field of virology, and first-hand experience in the virology laboratory.

INTRODUCTION

Viral disease is a complex process that involves a series of critical steps; these include entry of the virus particle into the host, infection of specific cells at the portal of entry, replication of the virus in the infected cells, and usually, the spread of the progeny virus particles within the infected host to other susceptible cells. Depending upon the nature of the particular viral agent, the deleterious effects for the host, if any, may result from cytolytic activity, cellular transformation, chronic cellular dysfunction, or the provocation of an injurious immunological response. Viruses contain 5 to 150 or more genes and their coordinated functioning is required for viral growth and, consequently, for survival of the virus in nature. Even though we do not generally understand the precise role of each viral gene product, it seems clear that viral infection and disease production requires proper functioning of most, if not all, viral genes and, in general, is not a consequence of any single viral gene product. In the case of oncogenic papovaviruses, transforming retroviruses and possibly adenoviruses, individual viral genes are thought to be responsible for the transforming properties of the virus.

Recombinant DNA experiments have already yielded new information about the structure and control of expression of genes in higher organisms that could not have been obtained by conventional techniques. DNA cloning provides unparalleled opportunities to explore the basic biology of animal and plant viruses. Virologists will be able to probe more deeply into the control of viral gene expression and discover phenomena of general cell biological significance; techniques will be more readily available to elucidate the sequence of viral nucleic acids, to shed light on the role of viral gene products in pathogenicity, and eventually, to understand the molecular biology of animal and plant viruses to the extent that some bacteriophages are now understood. It seems apparent that this new information will lead to a deeper understanding of viral diseases and to new ways of combating them. In the immediate future the ability to obtain useful amounts of pure viral genomes and subgenomic fragments that cannot be obtained by other means will provide scientists and physicians with invaluable and inexpensive diagnostic protein and nucleic acid reagents. In the more distant future it should be possible to use gene cloning techneques to obtain large amount of viral proteins; one practical benefit from such developments might well be effective and safe vaccines for control of diseases caused by hepatitis viruses, herpesviruses and influenza viruses, and many other viruses, both known and, as yet, unknown.

In addition to being able to clone viral genes in bacteria we are now able to envisage using certain animal viruses as vectors for the propagation of foreign genes in animal cells; a similar system for exploiting a plant virus, cauliflower mosaic virus, to clone foreign genes in plant cells may shortly become available. The chief importance of animal and plant virus vectors is that they can be used to carry genes into cells in which they may be fully expressed as well as propagated. By using specifically designed viral vectors it may eventually prove possible to deliver a specific gene to specific target cells; such techniques have obvious medical, economic, and agricultural applications but their realization will depend upon a great deal of basic research.

Viral Classification Systems and Recombinant DNA Experimentation

The group extensively discussed the current safety procedures for holding and handling in the laboratory certain animal viruses, in particular those likely to be used in cloning experiments in the foreseeable future, either as vectors or as the sources of the nucleic acids to be cloned. Inevitably the recommended safety measures for using animal (and plant) viruses in research vary from country to country. In the context of recombinant DNA research, which involves a novel set of circumstances, none of the available classifications of viruses according to the risks they pose is entirely satisfactory. A list of animal viruses was therefore prepared and the viruses were ranked according to their known hazard on the basis of: (a) the severity of human disease that they can cause, particularly in persons exposed in the laboratory; (b) their potential for infecting laboratory workers; (c) the risk that a laboratory infection might result in spread to the community; and (d) the impact such spread might have on the community or environment (Table 1). In this list four bacteria and one rickettsial agent with different pathogenic potentials have also been included as a frame of reference for the 22 viruses identified. Because of time constraints, many animal viruses, particularly those of agricultural and veterinary importance, were not included in the table. Although not comprehensive, the list contains most of those animal viruses that have been previously mentioned in the context of recombinant DNA experiments.

This list can also serve the very useful function of a reference scale, familiar to both microbiologists and clinicians, for expressing the degree of concern that a given conjectural hazard may engender, by comparison to a known biohazard.

Cloning Viral DNA's in E. Coli K12

The cloning of viral DNA's and cDNA's in *E. coli*K12 using EK1 and EK2 plasmid and lambda phage vectors was discussed in light of the conclusions of the Falmouth meeting that *E. coli* K12 is not pathogenic and does not efficiently colonize the vertebrate digestive tract (Gorbach, 1978). Not for want of trying, the participants were unable to envisage a sequence of events which could occur with significant probability that would allow *E. coli* carrying either whole DNA genomes of certain viruses or sub-genomic fragments of virtually any virus to lead to disease. The question was also raised as to whether or not, in the extremely remote possibility that all of the biological and physical containment barriers broke down, intestinal bacteria carrying cloned whole viral genomes might bypass the natural barriers to infection by the virus particle. As summarized in the following section, the group concluded that the probability that K12 organisms carrying viral DNA inserts could represent a significant hazard to the community was so small as to be of no practical consequence.

Risk Assessment Analysis for Cloning Viral DNA in E. Coli K12[1]

The following is a summary of the workshop discussions dealing with possible mechanisms of risk resulting from the cloning of genetic material of eukaryotic viruses (i.e., viruses of animals, plants, or lower eukaryotes) in *E. coli* vector systems.

We started with the extremely unlikely "worst case" assumption that a recombinant molecule containing eukaryotic viral genome sequences has in some way become established in wild type *E. coli* and has thereby become disseminated throughout the bowel flora of vertebrates. Given this hypothetical set of conditions, what consequences could be envisaged? For the purposes of analysis the discussions focused on two issues: first, the mechanisms by which the viral nucleic acid might gain access to cells of the host; and second, the nature of the inserted viral sequences.

Access of the viral genome to cells of the vertebrate host might conceivably result from virus particles formed within the bacterium or from release of viral nucleic acid into the proximity of, or into, host cells; this could occur either in the intestine or at the site of extraintestinal *E. coli* infection.

Production of infectious virus particles by bacteria carrying the recombinant DNA molecules was considered to be virtually impossible, regardless of the completeness of the viral genome or the nature of the eukaryotic virus from which it was derived. The basis for this high degree of assurance is in large part our great understanding of the molecular biology of virus replication. The nature of regulation of gene expression in prokaryotes is clearly different from that in eukaryotes as particularly exemplified by the absence in prokaryotes of RNA splicing mechanisms (Berget et al., 1977; Aloni et al., 1977; Lavi and Groner, 1977; Mellon and Duesberg, 1977; Chow et al., 1977; Klessig, 1977; Dunn and Hassell, 1977), RNA capping (Moore, 1966; Stavis and August, 1970; Blattner and Dahlberg, 1972; Maizels, 1973; Wei and Moss, 1975; Furuichi et al., 1975a; Keith and Fraenkel-Conrat, 1975; Abraham et al., 1975; Furuichi et al., 1975b; Dubin and Taylor, 1975; Perry and Scherrer, 1975; Moss and Koczot, 1976) and differences in messenger RNA biogenesis, polyadenylation (Kates and Beeson, 1970; Darnell et al., 1971; Mendecki et al., 1972; Philipson et al., 1971; Weinberg et al., 1972; Ehrenfeld and Summers, 1972; Pridgen and Kingsbury, 1972), and ribosomal binding sites (Shine and Dalgarno, 1974; Steitz and Jakes, 1975; Hagenbuchle et al., 1978). The expression of animal viral mRNA in *E. coli* translation systems is not accurate; in one well studied system, poliovirus, internal initiation signals are read which result in premature chain termination (Rekosh et al., 1970). Thus neither the synthesis of proper mRNA nor its translation into viral protein is likely to occur in *E. coli*.

Virus replication requires the regulated and coordinated function of multiple enzyme systems derived from both the host and the viral genomes; it seems most unlikely that any prokaryote contains the complement of enzymes required for the synthesis of an infectious animal virus. Animal viruses have evolved to be adapted to replication in eukaryotic cells; except for the instances where they have co-evolved to replicate in insect vectors, animal and plant viruses show a high degree of specificity for cells of a particular class or species of host cell or even a particular differentiated cell type. Further, there is no verified example of the replication of a virus of eukaryotes in bacteria, or conversely, the replication of a bacterial virus in any eukaryote. Consequently, since it was not considered possible for recombinant DNA containing a viral chromosome to produce intact virus particles in *E. coli*, the group felt secure in focusing on models involving host cell exposure to viral nucleic acid rather than viral particles.

In a model in which recombinant DNA-containing organisms are confined to the lumen of the intestine, the possibility of viral RNA or DNA gaining access to mucosal cells is extremely remote. First, the large amounts of nucleases in the intestinal contents would rapidly destroy the recombinant molecules (Maturin and Curtiss, 1977). Second, the efficiency of infection of cells by viral nucleic acid molecules, even in the presence of chemical potentiators, is extremely low, both in vitro and in vivo (Ellem and Colter, 1961; Amstey and Parkman, 1966; McCutchan and Pagano, 1968; Graham and van der Eb, 1973; Israel et al., 1978a). Third, since animal experiments indicate that polyoma viral DNA cannot initiate infection when administered by the oral or nasal routes (Israel et al., 1978a), it is likely that nucleic acids cannot infect across mucous membranes.

Consequently, the only model to which serious attention must be directed is one in which the hypothetical bacterium carrying the viral re-

[1]This portion of the report was prepared subsequent to the U.S.-EMBO Workshop by Drs. Martin and Rowe.

combinant DNA gains access to the body tissues. Two cases can be considered. With minor transgressions of the intestinal mucosa that allow brief penetration of organisms into the intestinal wall, lymphatics, or the portal circulation, the bacteria would be ingested by phagocytes. In phagocytes, bacteria are lysed and their nucleic acid is released in the nuclease-rich lysosomes; the effectiveness of the lysosomal enzymes virtually guarantees that no nucleic acid could survive to cause infection (Bensch et al., 1964; Carrara and Bernardi [1968]; Arsenis et al., 1970).

The second case, extraintestinal infection such as urinary tract or surgical wound infection, deserves serious consideration, but it must be pointed out that this would occur in only a fraction of individuals. In this context the consequences of the infection would be a function of the nature of the viral DNA inserted in the recombinant DNA molecule. We considered the following classes of DNA inserts: (1) subgenomic segments of nononcogenic viruses; (2) transforming segments of oncogenic viruses; (3) cDNA prepared from the genome of segmented RNA viruses; (4) cDNA copies containing the complete viral genome of non-segmented RNA viruses; and (5) complete DNA viral genomes. The workshop participants reached the conclusion that viral inserts should be thought in terms of three classes of risk: those for which there was no risk of a harmful outcome, and those for which a possibility of harm, however remote, could be envisioned. The latter were then divided into those scenarios which may well in reality be impossible, and those which are felt to be possible.

Those inserts for which the group could not construct any realistic harmful scenario were: (1) subgenomic segments of non-transforming RNA or DNA viruses; (2) cDNA copies from RNA viruses with segmented viral genomes and (3) cDNA's of the complete genome of negative strand RNA viruses.

(1) Subgenomic segments (meaning small portions of the viral genome lacking genes needed for replication of the virus) of non-transforming viruses are considered to be harmless because of the absence of any known example of an individual viral encoded protein which can act exogenously on cells. With the exception of some glycoproteins when added to cell cultures in high concentrations (Scheid and Choppin, 1974; McSharry and Choppin, 1978), viral proteins do not induce cell damage from without.

(2) Reverse transcripts of RNA viruses with segmented genomes cannot be envisaged as carrying any risk of producing infectious virus even when the cDNA is made from unfractionated nucleic acid preparations. It would be virtually impossible to ligate together a complete DNA copy, and if this ever did occur, we cannot envisage any way that the proper length RNA genome segments could be transcribed therefrom.

(3) Cloning of reverse transcripts of negative strand RNA viruses is viewed as being free of risk. With these viruses, the process of viral mRNA and genomic RNA synthesis is complex, and the Workshop participants could not envisage a set of circumstances in which RNA segments, transcribed from a DNA template, could eventuate in the synthesis of progeny virus. This is based on the fact that the nucleic acid of negative strand RNA viruses (either the plus or minus strand) has never been shown to be infectious for cells (Kingsbury, 1966; Baltimore et al., 1970; Wagner, 1975) presumably because of their unique molecular biology. Infection by negative strand viruses requires the activity of the virion associated transcriptase (Baltimore et al., 1970; Cormack et al., 1971; Szilagyi and Pringle, 1972); this enzyme catalyzes the synthesis of multiple (segmented) functional plus strand mRNA molecules from the input minus strand (Bratt and Robinson, 1967; Huang et al., 1970; Mudd and Summers, 1970; East and Kingsbury, 1971; Weiss and Bratt, 1976; Freeman et al., 1977). A polypeptide specified by one of these mRNAs then modifies the virion transcriptase to function as a replicase mediating the synthesis of a complete unsegmented plus strand of RNA, the template for synthesis of progeny minus strand RNA molecules. The minus strand has no messenger function (Huang et al., 1970; Grubman and Summers, 1973; Kingsbury, 1973; Morrison et al., 1974). Thus, to initiate the infectious process both a full length RNA transcript (to serve as template) and the segmented plus strand transcripts, capped for function as mRNA, would be required. This would necessitate either the transcription of both DNA strands by cellular RNA polymerase or the synthesis of a full length transcript as well as properly terminated, segmented transcripts from the minus strand DNA. These are obviously extremely unlikely events.

There were two classes in which it was considered that a risk scenario might be constructed but which might indeed be impossible; these involve the cloning of the transforming segments of DNA viruses or of transforming retroviruses, and the cloning of complete reverse transcripts of plus-strand RNA viruses. The model involving subgenomic, transforming segments postulates release of recombinant DNA molecules by lysis of *E. coli* in the tissues, and integration of the transforming gene segment into the DNA of host cells. While this eventuality cannot be ruled out, it was considered to have a very low probability in view of the inefficiency of transforming cells with viral nucleic acid (Aaronson and Martin, 1970; Graham et al., 1974; Abrahams et al., 1975) and the fact that integration of transforming DNA would occur in only a few cells in any one individual and, in the presence of competent immunosurveillance, would be most unlikely to result in a tumor (Habel, 1961; Sjogren, 1964; Sjogren et al., 1967; Costa et al., 1977). Transplantation studies with primary tumors and transformed tissue culture cells indicate that large numbers of cells are required to initiate tumor growth; in general, only after adaptation effected by long passage in animals do small numbers of cells produce tumors (Sjogren, 1964; Klein, 1975). In this regard, it should be noted that there is overwhelming evidence that humans are highly effective in averting tumorigenesis by DNA viruses. Man, and many other vertebrates as well, are infected repeatedly with DNA viruses (e.g. papovaviruses and adenoviruses) that contain the transforming regions discussed above (Huebner et al., 1954; Hilleman, 1957; Shah et al., 1973; Padgett and Walker, 1973; Brown et al., 1975; Shah and Nathanson, 1976). Some of these viruses are even known to induce tumors in laboratory rodents, yet, despite intensive search, none of these has been reproducibly associated with the etiology of any malignancy in humans (Mackey et al., 1976; Fiori and DiMayorca, 1976; Wold et al., 1978; Israel et al., 1978b).

The risk scenario whereby a cDNA transcript of a positive strand RNA virus leads to infection of the host was considered to have a finite but low probability. In this model, extraintestinal infection by the bacterium carrying the recombinant DNA results in the recombinant DNA molecule, or plus strand RNA transcripts thereof, entering into host cells with resultant productive viral infection. Neither carries a risk if the cDNA Insert is not a complete copy of the RNA genome. Unless a deliberate attempt is made to select full-length cDNA molecules for ligation to prokaryotic DNA vectors, the vast majority of recombinant DNA molecules will contain only a segment of viral specific DNA and, as a consequence, the likelihood of cloning the whole genome will be low.

The major block to synthesis of infectious RNA from the recombinant DNA molecule either in the prokaryotic or eukaryotic cell would be the inaccurate initiation of transcription. There is no reason to believe that the 5' terminus of the viral genome would generate a binding site for either prokaryotic or eukaryotic RNA polymerases following reverse transcription into DNA. Rather, RNA synthesis would be initiated either:

(1) Internally, by a random initiation process which would yield noninfectious molecules;

(2) Upstream, either at a prokaryotic promoter or at a random initiation site, in the eukaryotic cell which would generate a leader sequence lacking the appropriate recognition signals for binding to eukaryotic ribosomes (Shine and Dalgarno, 1974; Steitz and Jakes, 1975; Hagenbuchle et al., 1978). To produce infectious RNA from such a primary transcript, a site-specific cleavage would have to occur;

(3) At the 5' terminus by random initiation, but this undoubtedly would be an extremely rare event.

Any full-length plus strand transcripts synthesized in *E. coli* would have to escape degradation by extracellular ribonucleases following their release from bacteria and then initiate an infection in a sensitive cell, which, under the best conditions in the laboratory, is an extremely inefficient process (Ellen and Colter, 1961).

Further studies of this type of viral DNA insert would clearly be desirable.

The cloning of the complete genome of DNA viruses (including proviral forms of retroviruses) provides a scenario felt to carry a finite probability of risk, but this too is considered extremely low. Since viral DNA molecules are generally infectious, any situation in which the complete viral genome, without deletion or substitution, is excised from the recombinant molecule could conceivably lead to infection. In particular, circular DNA genomes, cleaved with a single cut restriction enzyme and ligated to a prokaryotic vector, provide such a condition; however, for accurate excision one must postulate the unlikely eventuality of the recombinant molecule being brought in contact with the same restriction enzyme used during the insertion of the DNA segment into the vector. Complete viral genomes could also be generated from a recombinant molecule in the case of oligomers of viral DNA inserts; a complete copy of the viral DNA could then be generated by intramolecular recombination. Full length linear DNA molecules inserted into a vector by oligo dA—oligo dT tailing or by addition of other linker molecules would be unlikely to excise accurately terminating genomes.

Given that it is conceptually possible to generate infectious molecules from recombinant DNA containing the complete genome of DNA viruses, it is important to assess the factors that would result in such infectious DNA causing disease. The probability that the excised molecules would infect host cells is of course relatively low in view of the inefficiency of infection by naked DNA (Ito, 1960; Burnett and Harrington, 1968; McCutchan and Pagano, 1968; Mayne et al., 1971; Graham and van der Eb, 1973; Sol and van der Noordaa, 1977). Another important factor in such a scenario is the susceptibility of neighboring cells to the virions produced by a cell infected with the infectious DNA. If the surrounding cells were not sensitive to the virus itself, the initially infected cell, producing virions, could not spread this infection to other cells or tissues; in the absence of virus spread there would be no risk other than the possible transformation of a small number of cells, in the case of oncogenic viruses, as discussed above. Only if the host was sensitive to the virus would spread occur; this would not be different from infection with the virus itself and would not generally represent a unique biohazard. Further study of this type of viral DNA insert would be extremely useful.

RECAPITULATION

It should be noted that these model scenarios were not constructed from an anthroipocentric viewpoint, but apply generally to any model in which vertebrates are colonized by the *E. coli* carrying the recombinant molecule.

To recapitulate, even assuming a worst case situation, this analysis leads us to conclude that cloning the subgenomic segments of nononcogenic viruses, the complete genome of negative strand RNA viruses, and any part of the genome of segmented viruses carries no risk of generating a biohazard. Second, it is possible to construct conceivable but extremely unlikely sets of circumstances resulting in a biohazard from the cloning of the transforming segment of oncogenic viruses. And third, it is possible to envisage feasible biohazard scenarios from cloning of the complete genome of DNA viruses or the entire genome of plus strand RNA viruses but even these carry little possibility of risk. When this analysis is combined with the immense unlikelihood of generating this worst case scenario in the first place, given good laboratory practice and the safety inherent in use of approved *E. coli* K12 host vector systems (Gorbach, 1978), the group felt strongly justified in concluding from available scientific information that viral genomes or fragments thereof, cloned in *E. coli* K12 using approved plasmid or phage vectors pose no more risk than work with the infectious virus or its nucleic acid and in most, if not all cases, clearly present less risk. In fact, the Workshop participants agreed that cloning of viral DNA in *E. coli* K12 may provide a unique opportunity to study with greatly reduced risks the biology of extremely pathogenic and virulent viruses.

The group also agreed that the cloning of cDNA copies of viroids in *E. coli* K12 should be postponed until more information is available about their molecular and cellular biology.

RECOMMENDATION

Based on these considerations the participants of the U.S.-EMBO Workshop concluded that the use of P2 (NIH Guidelines) or CI (Williams Report) containment measures, in conjunction with an EK1 host–vector system should provide adequate containment for cloning any viral genome or fragment thereof and recommended this as the minimum containment levels for recombinant DNA experiments involving eukaryotic viral DNA inserts. However, if the virus itself must be handled at higher levels of physical containment it seems prudent at the present time to use the more stringent containment conditions. It was emphasized that containment practices must include adequate training and the use of high quality microbiological technique.

EXPERIMENTS WITH EUKARYOTIC HOST-VECTORS

1. VERTEBRATE HOST-VECTOR SYSTEMS IN WHICH VIRAL DNA VECTORS ARE USED TO PROPAGATE OTHER DNA SEGMENTS

The Workshop participants endorsed the portion of the NIH guidelines that describes the features required for an animal virus to be used as a cloning vector (i.e. the first portion of section III.B.3.a. of the draft version, Revised NIH Guidelines for Recombinant DNA Research). The following is our recommended version of this section:

"Because this work will be done almost exclusively in tissue culture cells, which have no capacity for propagation outside the laboratory, the primary focus for containment is the vector; it should be pointed out that the risk of laboratory acquired infection as a consequence of tissue culture manipulations is very low. Given good microbiological practices, the most likely mode of escape of recombinant DNAs from a physically contained laboratory is carriage by an infected human; thus the vector with an inserted DNA segment should have little or no ability to replicate or spread in hmans. Further, a recombinant virus should not inadvertently pose a threat to any species.

For use as a vector in a vertebrate host cell system, an animal viral DNA molecule ideally should display the following properties:

(a) It should not consist of the whole genome of any agent that is infectious for humans or that replicates to a significant extent in human cells in tissue culture.

(b) It should be derived from a virus whose epidemiological behavior and biological properties are well understood.

(c) Its functional anatomy should be known—that is, there should be a clear idea of the location within the

molecule of: (i) The sites at which DNA synthesis originates and terminates. (ii) The sites that are cleaved by restriction endonucleases. (iii) the template regions for the major gene products.

(d) It should be defective when carrying an inserted DNA segment; that is, propagation of the recombinant DNA as a virus must be dependent upon the presence of a complementing helper genome. In almost all cases this condition would be achieved automatically by the manipulations used to construct and propagate the recombinants. In addition, the amount of DNA encapsidated in the particles of most animal viruses is defined within fairly close limits. The insertion of sizeable foreign DNA sequences, therefore, generally demands a compensatory deletion of viral sequences. It may be possible to introduce very short insertions (50–100 base pairs) without rendering the viral vector defective. In such a situation, the requirement that the viral vector be defective is not necessary.

If possible the helper virus genome should:

(i) Be integrated into the genome of a stable line of host cells (a situation that would effectively limit the growth of the vector recombinant to such cell lines) or

(ii) Consist of a defective genome, or an appropriate conditional lethal mutant virus, making vector and helper dependent upon each other for propagation.

However, neither of these stipulations is a requirement."

The group discussed at length the possibility that use of eukaryotic vectors under these conditions could pose a threat to the community or environment, but could not envisage a plausible set of circumstances whereby a risk to the community could develop. Given the extent of the genetic deletion required to satisfy the conditions stated above and in table 2, and the constraints that encapsidation places on the size of an inserted gene segment, the workshop participants saw no way that the experiments could generate a competent virus containing new genetic information sufficient to code for a functional gene product. The group tried to conceive of ways by which a recombinational event between the defective recombinant genome and the helper genome or the genome of an indigenous virus in an exposed laboratory worker might generate nondefective viral recombinants, but as mentioned, was unable to identify any.

The possibility that a defective recombinant genome might be maintained in nature through complementation by a helper virus was considered too unlikely to be a cause for concern, given the absence of any precedent in animal virology. The group considered the adeno-associated viruses, which are defective parvoviruses maintained in nature, to be a unique case in that they are known to integrate into host cells and to have at least 33 different potential helpers (human adenoviruses) available.

Having considered the use of the genomes of different DNA animal viruses to propagate DNA sequences from different sources, the group proposed the following recommendations:

TABLE 2

Viral vector DNA	Precaution level depending on source of foreign DNA		
	Prokaryotic	Eukaryotic	Viral
Polyoma virus—all or part	L or M	L	CbC
SV40—unconditionally defective by deletion of all or part of the sequences of any of the genes.	L or M	M	CbC
Adenoviruses 2 and 5—incapacitated by deletion of at least two capsid genes.	L or M	L	CbC
Murine adenovirus strain FL—all or part+	L or M	L	CbC
Herpes simplex virus—incapacitated by a large deletion	CbC	CbC	CbC

L corresponds to a containment level approximately equivalent to P2.

M corresponds to a containment level approximately equivalent to P3.

Prokaryotic sequences that are known not to contain toxigenic genes may be cloned in L conditions: otherwise M conditions should be used.

+Before this viral DNA can be used as a vector, further information is required about its host-range, particularly its interactions with cultured primate cells.

CbC These experiments should be assessed by the appropriate committees on a "Case by Case" (CbC) basis.

The group recommended that further work on the biological properties of recombinants formed between the genomes of two animal viruses should be carried out as a matter of some importance. The workshop participants discussed the possibility that new viral agents might be created with novel biological properties (e.g. host range, tissue tropism, pathogenicity) not found in either parent. Several model experiments were proposed to test this possibility. The group recommended that research in this area proceed cautiously with each case being considered on its individual merits and even if the model experiments suggest little cause for concern, continued careful surveillance of new recombinants should be maintained.

2. VERTEBRATE HOST-VECTOR SYSTEMS IN WHICH RECOMBINANT DNA'S ARE USED TO TRANSFORM CELLS

In these types of experiments viral DNAs carrying a foreign DNA segment will be used to transform cells in culture and in the process, integrate the recombinant DNA into the host cell chromosome. Some transformation systems are nonpermissive for progeny virus production and pose no possibility of producing laboratory infections. Other transformation systems are semi-permissive and, in addition to the appearance of transformed cells, allow the production of low titers of infectious virus.

When recombinant DNAs are used to transform cells which do not yield significant quantities of infectious virus (e.g., SV40 in murine cells, polyoma virus in rat or hamster cells, adenovirus 2 and 5 in rat cells) L conditions are generally sufficient; for viruses which do not infect humans, there need be no requirement that the vector be disabled. M conditions should be used if the system is semipermissive (i.e., virus is produced, in low titer) and the virus is capable of infecting humans (e.g., SV40 in human cell cultures). It was agreed that the DNA to be cloned must not be derived from another animal virus.

The use of viral genes as selective markers offers exciting and important possibilities for experiments involving cloning in eukaryotic cells. Possibly the best of such markers would be the herpes simplex virus thymidine kinase gene. We recommend that special emphasis be given to cloning in prokaryotic systems a small fragment (<KB) of herpes viral DNA which contains the sequences of this gene. Once available, the purified segment could be ligated to any chosen piece of DNA and cells transformed by the recombinant could easily be selected by virtue of the presence of thymidine kinase.

3. BACULOVIRUSES AS VECTORS

The group discussed the use of baculoviruses (large DNA-containing insect viruses) for cloning genes in invertebrate cells and concluded that our knowledge of the molecular and cellular biology of these viruses is too limited to allow any general recommendations. Proposals to use these viruses should be considered case by case. The group also recommended, however, that because of the potential exploitation of these viruses as biological pesticides (two have already been licensed for this purpose) and as vectors in recombinant DNA experimentation,

high priority should be given to studies of their basic virology, genetics, and molecular biology.

4. CAULIFLOWER MOSAIC VIRUS AS A VECTOR FOR CLONING GENES IN PLANT CELLS

The only known plant viruses which could serve as vectors for cloning genes in plants and plant cell protoplasts are Cauliflower Mosaic Virus (CaMV) and its close relatives, which have relaxed circular double stranded DNA genomes with a molecular weight of 5×10^6. The genomes of these viruses are not known to integrate into host chromosomes, or to pick up cellular genes. CaMV is spread in nature by aphids, in which it survives for a few hours. Spontaneous mutants of CaMV that are not transmitted by aphids arise frequently; these mutants fail to make a transmission factor essential for aphid transmission.

The viruses in the CaMV group have narrow host ranges and are relatively difficult to transmit mechanically to plants. For this reason, they are most unlikely to be accidentally transmitted from spillage of purified preparations of the virus,

The workshop participants recommended that for use as a vector with intact plants, a strain should be selected which is not transmitted by aphids. The ability to produce local lesions in an appropriate host would be an advantage in maintaining the integrity of the strain. The plants should be grown in either a greenhouse or plant growth cabinet which is insect-proof. Soil, plant pots and unwanted infected plant materials should be removed from the greenhouse or cabinet in sealed insect proof containers and sterilized. It is not necessary to sterilize run-off water from the infected plants as this is not a plausible route for secondary infections. Infected plant materials to be used for further research, which have to be removed from the greenhouse or cabinet, should be maintained under insect proof conditions. These measures provide an entirely adequate degree of containment and are similar to those required in many countries for licensed handling of "exotic" plant viruses.

CaMV or its DNA may also be useful as a vector to introduce genes into plant protoplasts. The fragility of plant protoplasts combined with the properties of CaMV mentioned above provides adequate safety. Since the group envisaged no risks to the environment from use of the CaMV-protoplast system, no special containment was recommended.

TABLE 1.—*Risk of illness in laboratory workers and of spread of viruses to the environment*

Agent	Genome	Severity of disease in adult humans	Risk of laboratory infection	Transmission into community or environment	Community or environmental impact
Lassa fever virus	RNA—negative strand, segmented.	4+	H	L to H	H.
Variola virus	DNA—linear, double stranded.	4+	H [1]	L to H	H.
Yersinia pestis (Plague bacterium)*.		4+	H	L to H	L to H.
Yellow fever virus (wild type).	RNA—positive strand	3+	H [2]	Dependent on presence of vector (H).	
Rickertsia prowazeki (typhus agent)*.		3+	H	L	0.
Hepatitis B virus	DNA—circular, partially single stranded.	3+	L to H	L	0 to L.
Vibrio comma (cholera bacterium)*.		2+ to 3+	L	L	0 to L.
Influenza virus (novel epidemic strain).	RNA—negative strand, segmented.	1+ to 2+	L	H	H.
Epstein-Barr virus	DNA—linear, double stranded.	2+	L	L	0.
Polio virus (wild)	RNA—positive strand	0 to 3+	L	L	0 to L.
Coxsackie viruses	do	0 to 2+	L	L	0 to L.
Herpes simplex virus	DNA—linear, double stranded.	1+	L	L	0.
*Shigella sonnei**.		1+	L	L	0.
Vesicular stomatitis virus	DNA—negative strand	1+ to 2+	H	L	[3]
Adenovirus 2 or 5	DNA—linear, double stranded.	0 to 1+	0 to L	L	0.
Adeno-SV40 hybrid viruses (nondefective).	do	do	do	L	0.
Vaccinia virus	do	1+	L	L	0.
Human Papilloma virus	DNA—circular, double stranded.	1+	L	L	L to 0.
Influenza virus (PRB)	DNA—negative strand, segmented.	0	0	0	0.
Bacteroides sp.*		0 to 1+	0	L	0.
Adeno-associated virus	DNA—linear, single stranded.	0	L	L	0.
BK virus	DNA—circular, double stranded.	0	L	0	0.
Simian virus 40 (SV40)	do	0	L to H(?)	0 to L(?)	0.
Sindbis virus (Lab. adapted)	DNA—negative strand	0 to 1+	0	Dependent on presence of vector (0-L)	
Semliki Forest Virus	do	do	0	Do.	
Reoviruses	DNA—double stranded, segmented.	0	0 to 1+	0	0.
Rous sarcoma virus	RNA (Retrovirus)	0	0	0	0.
Murine leukemia sarcoma viruses.	do	0	0	0	0.
Feline leukemia viruses	do	0	0	0	0.
Polyoma virus	DNA—circular, double stranted.	0	0	0	0.

*Non-viral agents.
H=High probability; L=Low probability.
[1] If not vaccinated.
[2] L for human; H for certain apimals.

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[FR Doc. 78-8362 Filed 3-30-78; 8:45 am]